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LOCALIZATION OF ENDOTHELIN IN THE BLOOD-BRAIN INTERPHASE IN RAT HIPPOCAMPUS AFTER GLOBAL CEREBRAL ISCHEMIA

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Electron microscopic immunocytochemical evaluation of localization and distribution of endothelins 1, 2 and 3 in the CA1 hippocampal sector in rats submitted to 10 min global cerebral ischemia was performed. The studies were done in different postischemic periods (10 min, 3, 6, 12 and 24 h) with postembedding immuno-gold technique for electron microscopy. Endothelin-like immunoreactivity was found in endothelial cells of hippocampal microvessels and in astrocytes, microglia, macrophages and in some axonal endings.

The most pronounced changes appeared 24 h after ischemia. At that period all structural elements of blood-brain interphase: endothelium, basal membrane and perivascular astrocytic processes showed intensive endothelin-like immunoreactivity. Especially marked immunoreactivity was found in macrophages appearing in the proximity of microvessels.

It has been stressed that ischemia-induced increased content of endothelin may play an important role in the pathogenesis of postischemic tissue abnormalities.

Key words: *endothelin, immunocytochemistry, cerebral ischemia.*

It is well known that endothelins (ETs), 21 amino acid peptides, play an important role in a number of pathological conditions involving various body organs and systems.

There exist three separate, well characterized chemically and pharmacologically ET-isoforms, occurring both in humans and other mammalian species. They are known as ET-1, ET-2, and ET-3. The "vasoactive intestinal contractor" or endothelin β is the fourth distinct isoform (Battistini et al. 1993). Endothelins are known to be present in different tissue elements of the central nervous system. ET-1, one of the most potent endogenous vasoconstrictor substance is produced by endothelial cells (Yanagishawa et al. 1988; Yanagishawa, Masaki 1989; Yoshimoto et al. 1990). Endothelial ET-2 may be released in response to thrombin, vasopressin, angiotensin II or transforming growth factor β (Yanagishawa, Masaki 1989; Shini, Vanhoutte 1991). There are immunocytochemical evidences of neuronal and glial localization of ET-2 and ET-3 (Marsault et al. 1990; Fuxe et al. 1991). Two different ET receptor subtypes have been identified: ET_A and ET_B, both of them coupled with phospholipase C (Sakurai et al. 1990). ET_A-receptor subtype reveals high affinity to ET-1 and relatively low to ET-3 (Arai et al. 1990). It is responsible for the action of endothelins on vascular smooth muscle cells and on atrial cells (Vigne et al. 1990). Non-selective ET_B receptor is mainly expressed in neurons and astrocytes (McCumber et al, 1990; Marsault et al. 1990). It has been shown that ET-2 is also produced in many other types of cells, among others by epithelial cells, leukocytes, macrophages, some cancer cell lines and others. In addition, endothelins are mitogenic for vascular smooth muscle cells and potentiate growth factor–stimulated DNA synthesis in these cells (Takuwa et al. 1989; Brown, Littlewood 1989; Bobik et al. 1990).

The role of ETs in the central nervous system has been characterized by Sakurai et al. (1990), Lee et al. (1990), Niwa et al. (1991) and others. It has been shown that ETs may behave in the central nervous system as neurotoxic factors and participate in neuronal pathology caused by excitatory amino acids (Cintra et al. 1989; Fuxe et al. 1989). The role of the endothelins in the development of neuronal death due to cerebral ischemia was recently demonstrated by Fuxe et al. (1992) and Yamashita et al. (1992).

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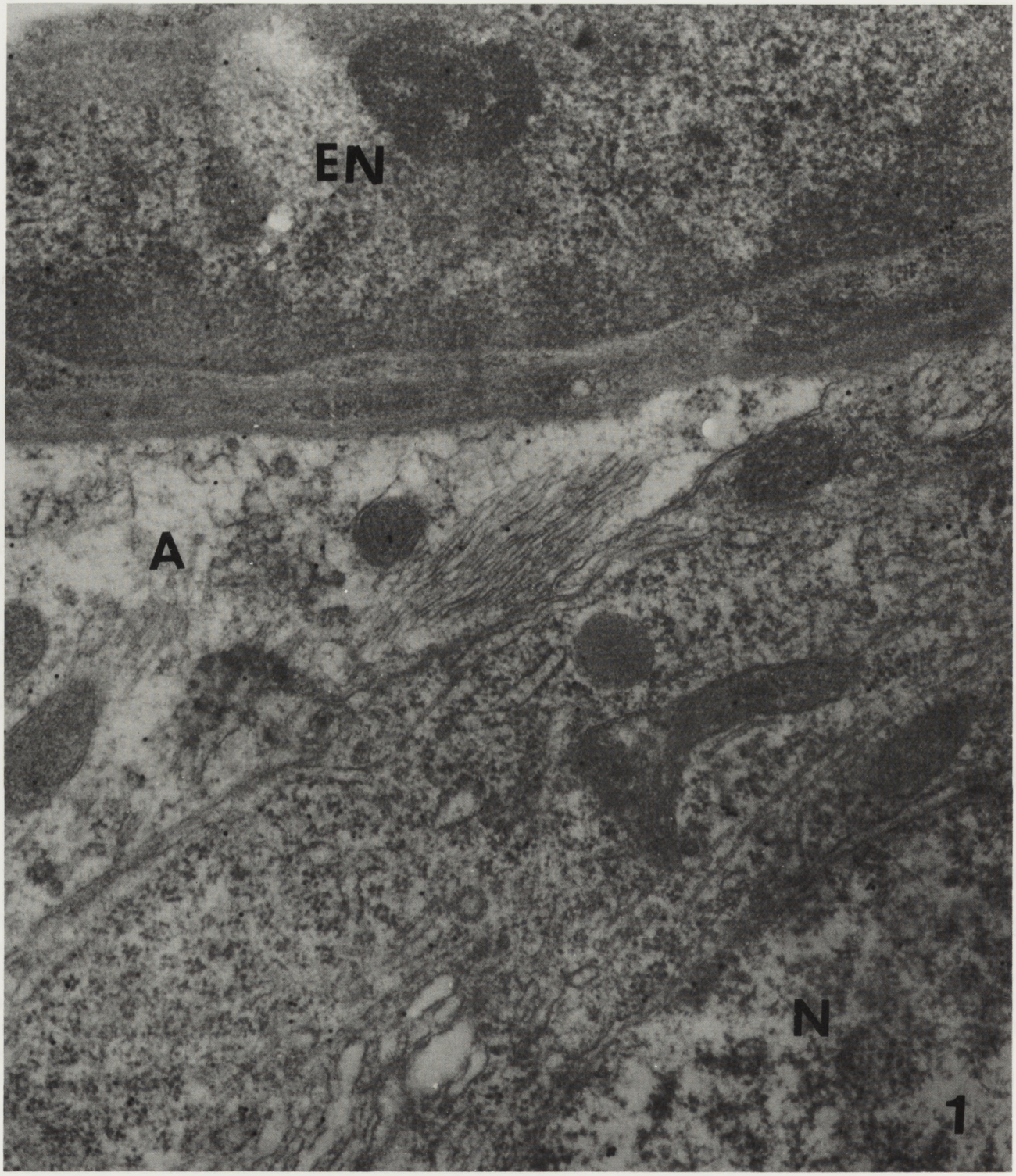

Fig. 1. Control animal. Weak immunoreactivity, visualized in the form of dark dots representing 10 nm gold particles, is present in endothelial cell (EN), perivascular astrocyte (A) and in neighbouring neuron (N). × 15000

The aim of present study was to characterize changes in endothelin-like immunoreactivity in CA1 sector of Ammon's horn in the condition of global cerebral ischemia in rats. The CA1 hippocampal sector is known of the selective vulnerability of its pyramidal neurons to ischemia (Smith et al. 1984) and some peculiarities in the reaction of its vascular network to the ischemic incident (Imdahl, Hossmann 1982; Gadamski, Mossakowski 1992).

Material and methods

The experiments were performed on adult male Wistar rats, sixteen of which were subjected for 10 min to global cerebral ischemia due to experimentally induced cardiac arrest, according to the met-

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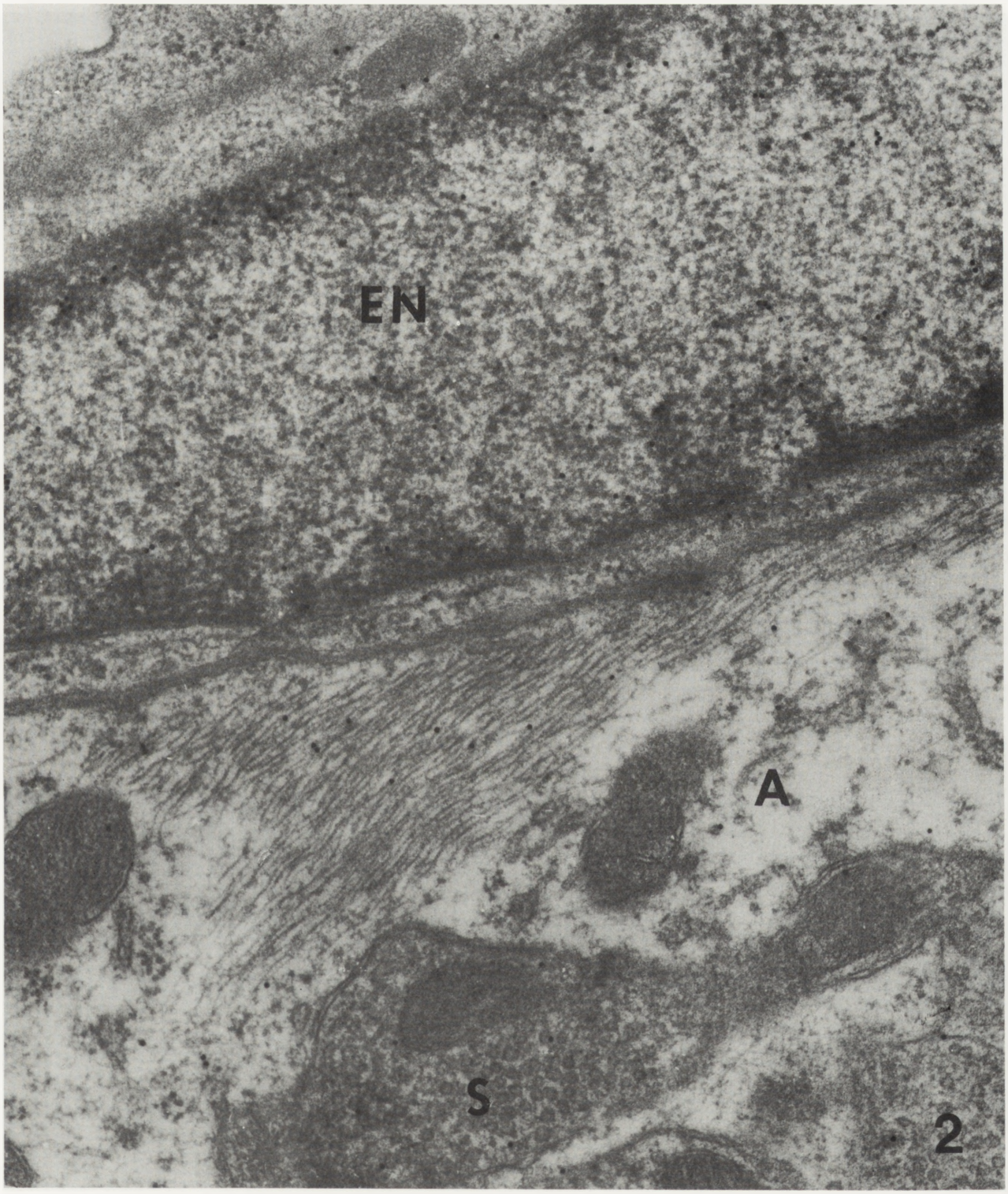

Fig. 2. Control animal. Weak immunoreactivity visible in endothelial cell (EN). More gold particles are present in perivascular astrocytic process (A), some are seen in nerve terminals (S). × 20000

hod described originally by Korpachev et al. (1982). Detailed characteristics of the experimental procedure and basic pathophysiological observations were presented in previously published papers (Mossakowski et al. 1986; Kapuściński 1987). Four animals not submitted to cerebral ischemia served as control group. The experimental animals were sacrificed 10 min, 3, 6, 12 and 24 h after cerebral ischemia. Both control and experimental animals were anesthetized with ether and perfused transcardially with physiological saline solution (1 min) followed by solution consisting of 0.2% glutaraldehyde and 2.5% paraformaldehyde in 0.1 M sodium phosphate buffer (PBS), pH 7.4 for 30 min. Blocks of tissue, taken from hippocampal CA1 sector, rinsed for 2 h in PBS, treated with 1%

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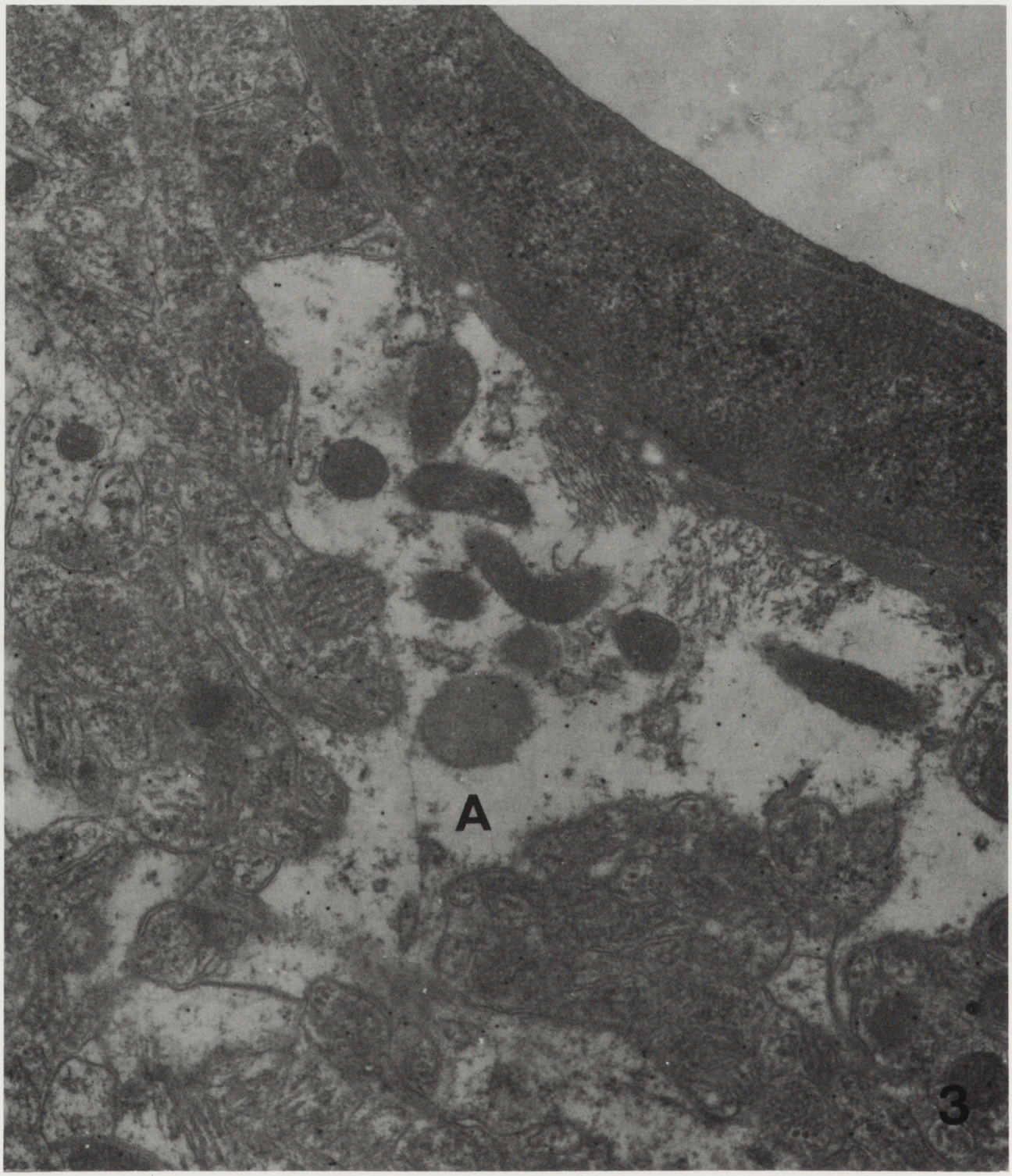

Fig. 3. Experimental animal, 10 min after ischemia. Relatively strong immunoreactivity is visible in swollen perivascular astrocytic process (A). × 12 000

osmium tetroxide for 1 h, dehydrated in series of graded alcohol solutions were finally embedded in Epon.

Ultrathin sections were treated according to post-embedding immuno-gold procedure. They were mounted on formvar-coated golden grids, floated for 10 min on 10% H_2O_2 and then rinsed in PBS for 15 min and exposed for 15 min to 5% bovine serum albumin in PBS. Monoclonal antibody to endothelin 1, 2 and 3 (human) (Biogeneses, U.K. Cat. no 4113-0957) was diluted 1:100 in PBS and applied on sections for 3 h in 37°C. Subsequently the grids were washed in PBS for 30 min and exposed to goat antirat IgG (H+L) (Human ABS) conjugated with colloidal gold particles, 10 nm in diameter (Janssen Pharmaceutica, Beerse, Belgium)

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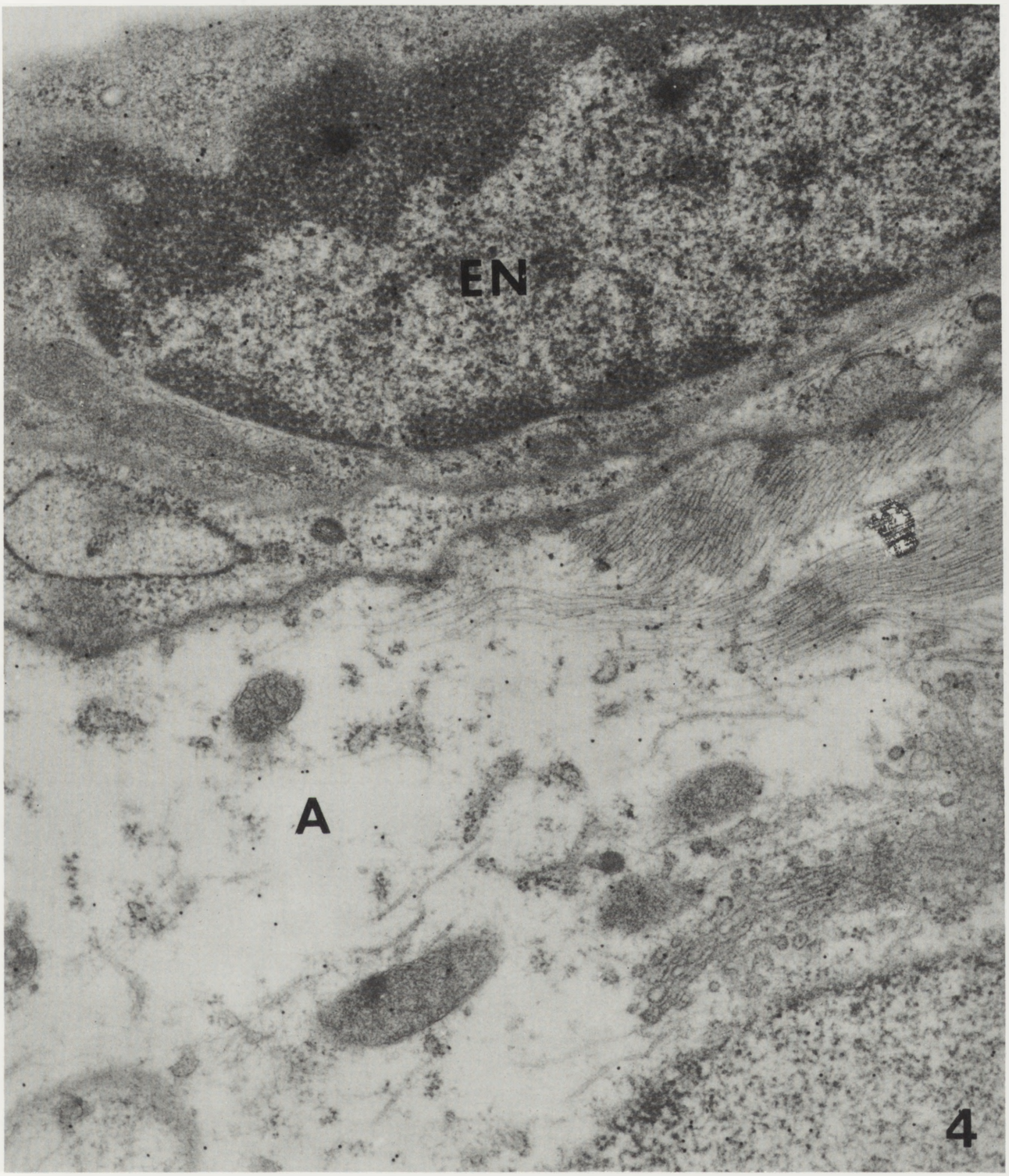

Fig. 4. Experimental animal, 12 h after ischemia. Relatively high immunoreactivity both in endothelial cell (EN) and in perivascular swollen astrocytes (A). Some dots are visible over basal vascular membrane. × 12000

diluted 1:50 in PBS. After incubation for 30 min in darkness the grids were washed in PBS for 15 min and then in distilled water for the next 15 min. At the end they were air-dried, stained for 10 min with 4.7% uranyl acetate and for 2 min with lead citrate.

Control sections were prepared using normal murine serum instead of anti-endothelin antibody. Sections were examined and photographed using JEOL 1200 EX electron microscope.

Results

Control animals.

In CA1 hippocampal sector ET-like immunoreactivity was present in endothelial cells of all visible

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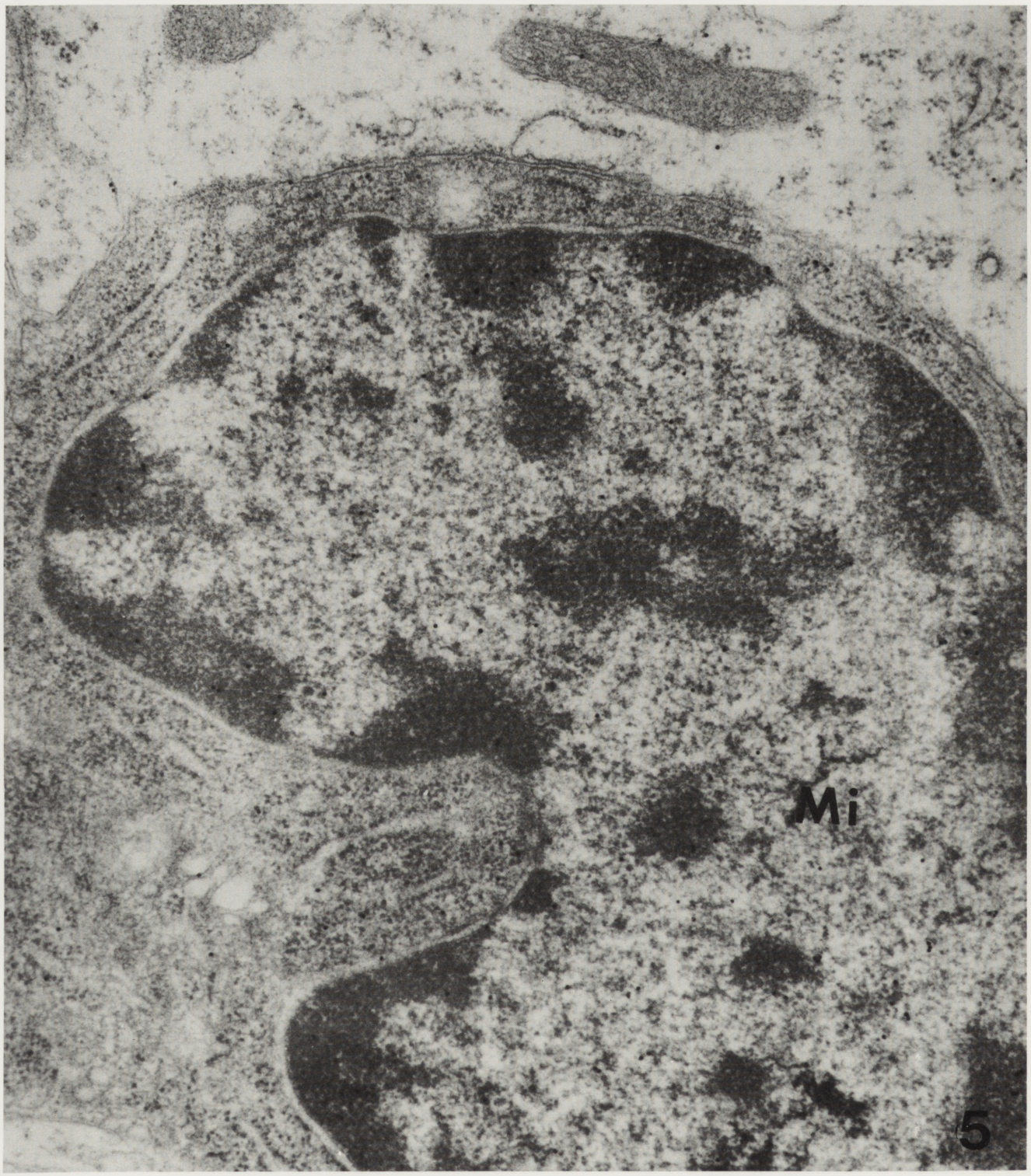

Fig. 5. Experimental animal, 12 h after ischemia. Black dots indicating endothelin-like immunoreactivity in microglial cell (Mi). × 15000

blood vessels. The gold particles were visible predominantly over the cell nuclei with much weaker reaction over the cell cytoplasm (Fig. 1). ET-specific labelling was detectable in all glial cells. Astrocytic cell bodies and processes exhibited moderate immunoreactivity. Higher ET-like immunoreactivity occurred in perivascular processes of astrocytes (Fig. 1, 2). A few nerve terminals were immunoreactive (Fig. 2). The general impression was that in normal rats hippocampus the ET-like immunoreactivity prevailed in astroglial cells, being weaker both in endothelial cells and nerve elements.

Experimental animals

Ten minutes after ischemia ET-like immunoreactivity in great majority of microvessel endothelial

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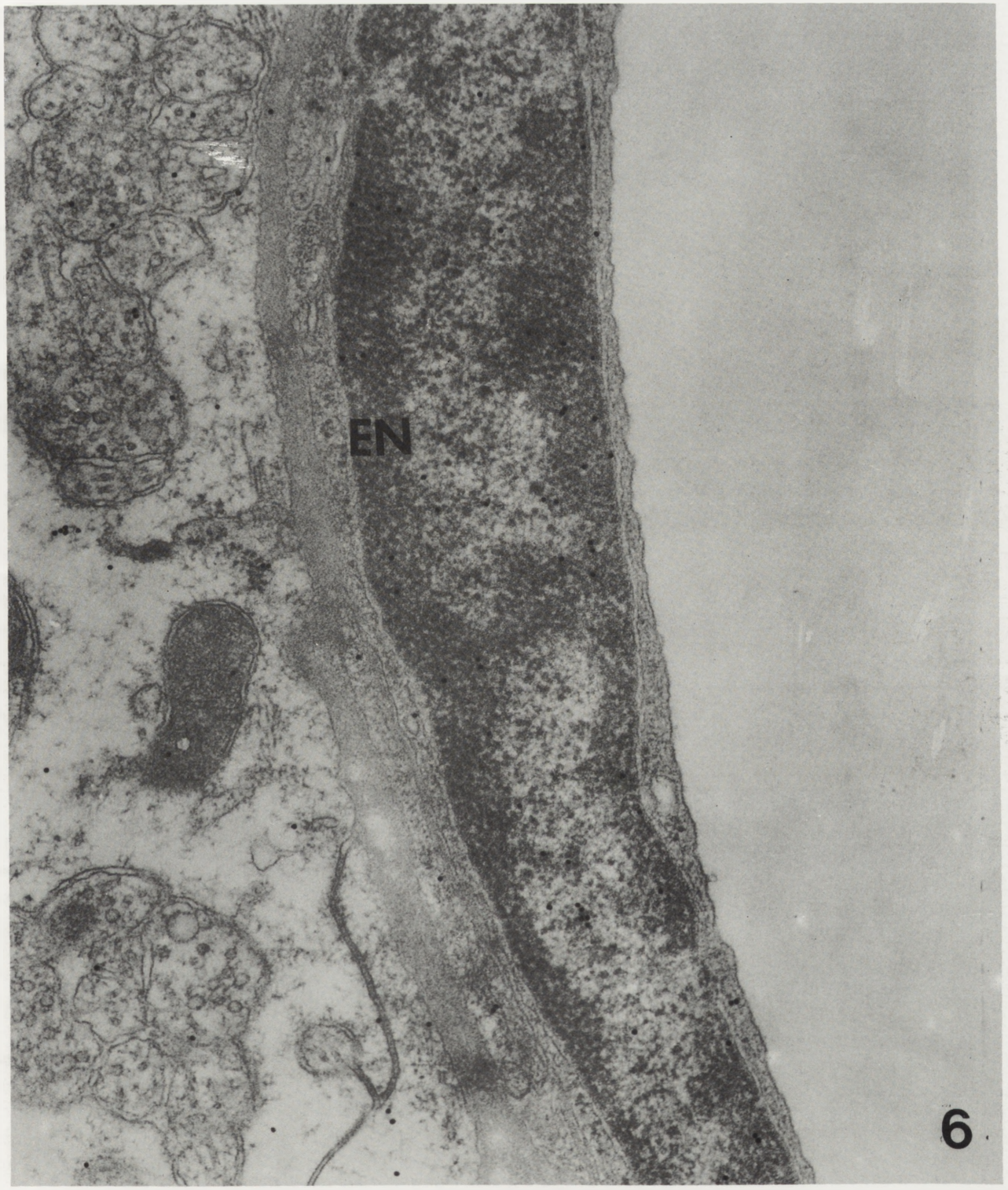

Fig. 6. Experimental animal, 24 h after ischemia, Numerous dots of colloidal gold are present in unchanged endothelial cell (EN). × 20000

cells was comparable to that seen in the control animals. Sometimes, the density of gold particles was higher in some swollen endothelial cells. There was no difference in the intensity of ET-like immunoreactivity in perivascular astrocytic processes as compared to control animals. The immunocytochemical reaction was strong in all astrocytes (Fig. 3).

Three and six hours after ischemia the distribution and intensity of ET-like immunoreactivity did not show any noticeable differences as compared with the above described picture.

Twelve hours after ischemia ET-like immunoreactivity in endothelial cells visible in CA1 hippocampal sector was higher than in the controls. The majority of glial cells perikarya and processes were swollen, but the ET-like immunoreactivity was visib-

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Fig. 7. Experimental animal, 24 h after ischemia. High endothelin-like immunoreactivity in macrophage (M) located in the vicinity of the blood vessel. × 12000

le in them (Fig. 4). High intensity of the reaction was seen in microglial cells (Fig. 5).

Twenty four hours after ischemia strong ET-like immunoreactivity was seen in endothelial cells of most microvessels, these being more marked in vessels of larger caliber (Fig. 6). Accumulation of gold particles was visible even over ultrastructurally changed endothelial cells. Similar as in normal conditions, density of diffusely spread gold particles was higher over cellular nuclei than over cytoplasm. High labelling characterized astrocytic processes both in neuropil and in perivascular localization. However, the strongest labelling was detected in macrophages and in microglial cells. The great number of these cells appeared in the vicinity of blood vessels in this stage of postischemic period (Fig. 7).

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Discussion

Our observations indicate that global cerebral ischemia evokes changes in content and distribution of endothelins in hippocampal CA1 sector. The most interesting findings concern dynamics of these changes and involvement of various nerve tissue elements.

At early stages of the postischemic period (10 min – 6 h) ET-like immunoreactivity in microvessel endothelial cells was very weak (almost on the limits of the immunocytochemical detection), it started increasing 12 h after ischemia to reach the highest intensity at 24th h of the recirculation.

It has been shown previously that endothelial cells produce both endothelium-derived relaxing factor (EDRF) or nitric oxide, causing relaxation of underlying smooth muscle cells, and endothelins, revealing an opposite activity, in response to various physiological and damaging hemodynamic conditions and chemical substances (Vanhoutte 1987; Tomimoto et al. 1994). EDRF is considered to be involved in rapid local control of vascular tone, whereas endothelins seem to contribute in a long-term regulation. The relatively low ET-like immunoreactivity (probably slow production of ETs) suggests that peptides are more likely to participate in long-term regulatory processes than in acute responses (Shini, Vanhoutte 1991). Our observations support this opinion. Changes in the ET-like immunoreactivity in microvascular endothelial cells were rather late phenomenon. This may suggest their influence on the local circulation abnormalities appearing in later phases of the postischemic condition. This seems to be of special interest in the context of peculiar microcirculation abnormalities described by Imdahl and Hossmann (1982) in CA1 sector in Mongolian gerbils after short-term forebrain ischemia. It consisted in reduced volume of circulating blood, persisting in CA1 sector much longer as compared with neighbouring Ammon's horn sectors and other brain regions. It seems worth of stressing that in the condition of our experiments increased ET-like immunoreactivity has been appearing in endothelial cells revealing ultrastructural abnormalities of different intensity, being much weaker in normally looking endothelium. This finds support in observations of Yoshimoto et al. (1990), who demonstrated that injured endothelial cells are producing more endothelin than normal ones.

Our immunocytochemical observations provide additional evidence that ET-like peptides are formed in astrocytes. Moreover, there was an increase in the intensity of ET-like immunoreactivity in practically all hippocampal astrocytes during the whole postischemic period, starting already from 10th minute of recirculation. The most intense reaction concerned perivascular astrocytic processes. It seems justified to postulate that ischemia-induced increased production of endothelins in astrocytes may be an important factor influencing regional cerebral blood flow and function of the blood brain barrier.

The most striking finding of our investigations was appearance of high ET-like immunoreactivity in microglia and macrophages in the proximity of the blood vessels. The structural elements of their walls are potential targets for the action of microglia-macrophage-derived endothelins. Intense accumulation of highly immunoreactive microglial cells and macrophages in the vicinity of blood vessels was observed at 24th h following brain ischemia. It seems that microglia-macrophage-derived endothelins may contribute significantly to the total pool of these peptides in the brain tissues, in the conditions of the increased content of these cells, potentiating the physiological role of endothelins, both as vasoconstrictors and neurotransmitters or neuromodulators.

Overall increase of ET-like immunoreactivity of both endothelial and astrocytic as well as microglial-macrophagic origin observed in CA1 hippocampal sector in different phases of postischemic condition is strongly suggestive that endothelins, influencing regional cerebral blood flow, may play an important role in pathogenesis of the ischemia-induced functional and structural brain alterations.

Lokalizacja endoteliny w interfazie naczyniowo-tkankowej w hipokampie szczura po całkowitym niedokrwieniu mózgu

Streszczenie

Przeprowadzono immunocytochemiczną ocenę rozmieszczenia endoteliny w sektorze CA1 hipokampa szczurów po 10 min całkowitym niedokrwieniu mózgu. Badania wykonano po upływie 10 min, 3, 6, 12 i 24 godz po niedokrwieniu przy użyciu dostosowanej do mikroskopu elektronowego techniki immunocytochemicznej, wykorzystującej złoto koloidalne.

Immunoreaktywność dla endoteliny wykazano nie tylko w komórkach śródbłonka naczyniowego, lecz również w astrocytach, mikrogleju, makrofagach i w niektórych zakończeniach aksonalnych.

Największą intensywność zmian wykazano po upływie 24 godz po niedokrwieniu. W okresie tym immunoreaktywność występowała we wszystkich elementach złącza naczyniowo-tkankowego: śródbłonkach, błonach podstawnych naczyń i w okołonaczyniowych astrocytach. Szczególnie intensywny odczyn występował w makrofagach gromadzących się w sąsiedztwie naczyń.

Wydaje się, że zależne od niedokrwienia zwiększenie zawartości endoteliny może wskazywać na jej ewentualny udział w patogenezie poischemicznych uszkodzeń mózgu.

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References

1. Arai H, Hari S, Aramori I, Ohkabo H, Nakamishi S: Cloning and expression of a c-DNA encoding and endothelin receptor. Nature, 1990, 348, 730-732.
2. Battistini B, D'Orleans-Juste P, Sirois P: Biology of disease. Endothelins: circulating plasma levels and presence in other biologic fluids. Laboratory Invest, 1993, 68, 600-628.
3. Bobik AA, Grooms A, Millar JA, Mitchell A, Grinpukle S: Growth factor activity of endothelin on vascular smooth muscle. Am J Physiol, 1990, 258, c 408-415.
4. Brown KD, Littlewood CJ: Endothelin stimulates DNA synthesis in Swiss 3T3 cells. Synergy with polypeptide growth factors. Biochem J, 1989, 263, 977-980.
5. Cintra A, Fuxe K, Anggard E, Tinner B, Staines W, Agnati LF: Increased endothelin-like immunoreactivity in ibotenic acid-lesioned hippocampal formation of the rat brain. Acta Physiol Scand, 1989, 137, 557-558.
6. Fuxe K, Cintra A, Andbjer A, Anggard E, Golstein M, Agnati LF: Centrally administreted endothelin-1 produced lesions in the brain of the male rat. Acta Physiol, 1989, 137, 155-156.
7. Fuxe K, Kurosawa N, Cintra A, Hallstrom A, Goiny M, Rósen L, Agnati LF, Ungerstedt U: Involvement of local ischemia in endothelin-1 – induced lesions of the neostriatum of the anesthetized rat. Exp Brain Res, 1992, 88, 131-139.
8. Fuxe K, Tinner B, Staines W, Hemsen A, Hersh J, Lundberg JM: Demonstration and nature of endothelin-3-like immunoreactivity in somatostatin and choline acetyltransferase immunoreactive nerve cells of the neostriatum of the rat. Neurosci Lett, 1991, 123, 107-111.
9. Gadamski R, Mossakowski MJ: Asymmetric damage of the CA1 sector of Ammon's horn after short-term forebrain ischemia in Mongolian gerbils. Neuropatol Pol, 1992, 30, 209-219.
10. Imdahl A, Hossmann A: Morphometric evaluation of postischemic capillary perfusion in selectively vulnerable areas of gerbil brain. Brain Res, 1982, 239, 57-69.
11. Kapuściński A: Cerebral blood flow in experimental model of clinical death (in Polish). Neuropatol Pol, 1987, 25, 287-298.
12. Korpachev G, Lysenkow SP, Tiel LZ: Modelling of clinical death and postresuscitation disease in rats (in Russian). Patol Fizjol Exp Ter, 1982, 3, 78-80.
13. Lee ME, De la Monte NG, Bloch KD, Quertermous T: Expression of the potent of the vasoconstrictor endothelin in the human central nervous system. J Clin Invest, 1990, 86, 141-147.
14. McCumber MW, Ross CA, Snyder SH: Endothelin in brain: receptors, mitogenesis, and biosynthesis in glial cells. Proc Natl Acad Sci USA, 1990, 87, 2359-2362.
15. Marsault R, Vigne P, Breittmayer JP, Frelin C: Astrocytes are target cells for endothelins and sarafotoxin. J Neurochem, 1990, 54, 2142-2144.
16. Mossakowski MJ, Hilgier W, Januszewski S: Pathomorphology of the central nervous system in experimental postresuscitation syndrome (in Polish). Neuropatol Pol, 1986, 24, 471-489.
17. Niwa M, Kawaguchi T, Yamashita K, Maeda T, Kurihara M, Kataoka Y, Ozaki M: Specific ^{125}J-endothelin-1-binding sites in the central nervous system. Clin Exp Hypertens, 1991, A13, 799-806.
18. Sakurai T, Yanagishawa M, Takuwa Y, Miyazaki H, Kimura S, Goto K, Masaki T: Cloning of a c-DNA encoding a non-isopeptide-selective subtype of endothelin receptor. Nature, 1990, 348, 732-735.
19. Shini VB, Vanhoutte PM: Endothelin-1: a potent vasoactive peptide. Pharmacol Toxicol, 1991, 69, 303-309.
20. Smith ML, Auer RN, Siesjo BK: The density and distribution of ischemic brain injury in the rat following 2-10 min of forebrain ischemia. Acta Neuropathol (Berl), 1984, 64, 319-320.
21. Takuwa NY, Takuwa M, Yanagisawa M, Yamashita K, Masaki T: A novel vasoactive peptide endothelin stimulates mitogenesis throught inositol lipid turnover Swiss 3T3 fibroblast. J Biol Chem, 1989, 264, 7856-7861.
22. Tomimoto H, Akiguchi J, Wakiba H, Nalamura S, Kimura J: Histochemical demonstration of membranes localization of endothelial nitric oxide synthase in endothelial cells of the rat brain. Brain Res., 1994, 667, 107-110.
23. Vanhoutte PM: Endothelium-dependent contractions in arteries and veins. Blood-Vessels, 1987, 24, 141-144.
24. Vigne P, Breittmayer JP, Marsault R, Frelin C: Endothelin stimulates phosphatidylinositol hydrolisis and DNA synthesis in brain capillary endothelial cells. Biochem J, 1990, 266, 415-420.
25. Yamashita K, Kataoka Y, Niwa M, Shimematsu K, Himeno A, Koizumi S, Taniyama K: Increased production of endothelins in the hippocampus of stroke-prone spontaneously hypertensive rats following transient forebrain ischemia: histochemical evidence. Cellular Molecular Neurobiol, 1993, 13, 15-23.
26. Yanagishawa M, Kurihawa H, Kimura H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T: A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature, 1988, 332, 411-415.
27. Yanagishawa M, Masaki T: Endothelin, a novel endothelium-derived peptide: pharmacological activities, regulation and possible roles in cardiovascular central diseases. Biochem Pharmacol, 1989, 38, 1877-1883.
28. Yoshimoto S, Ishizaki Y, Kurihawa H, Sasaki T, Yoshizumi M, Yanagishawa M, Yazaki Y, Masaki T, Takakura T, Murota S: Cerebral microvessels endothelium is producing endothelin. Brain Res, 1990, 580, 283-285.

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